



# Delivery of cytotoxic biologics to target cells using nanosyringes that self-assemble during production

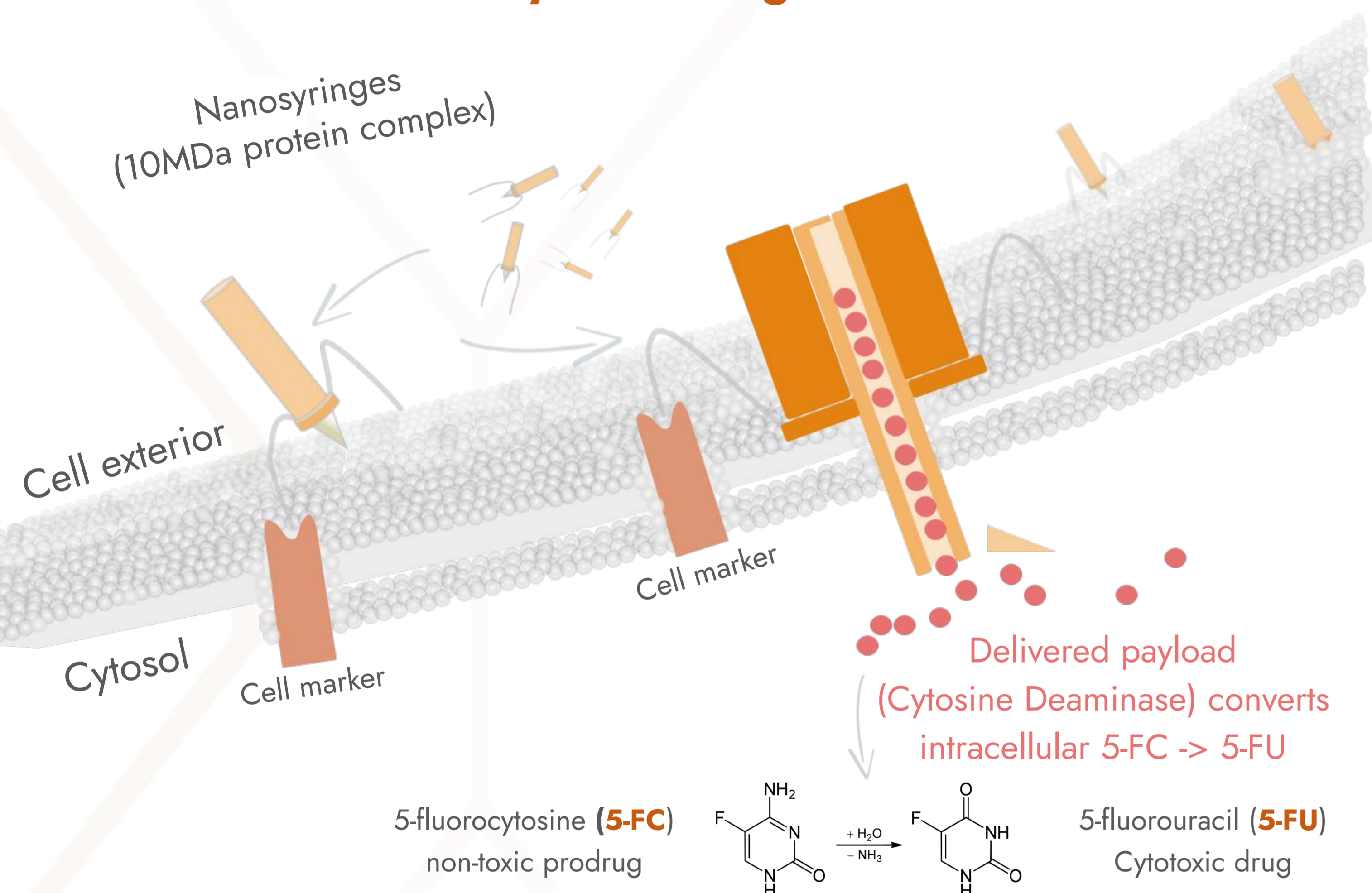
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## Introduction

Suicide gene therapy utilizing the cytosine deaminase/5-fluorocytosine (CD/5-FC) system can induce efficient anti-tumour effects. Some of the limitations associated with this approach include low transduction efficiency and targetability of the gene delivery vectors. In this proof-of-concept study we leverage the nanosyringe targeted protein delivery technology to deliver *E. coli*-derived cytosine deaminase (CD) to murine macrophages. Once delivered, the active CD converts intracellular 5-FC prodrug into 5-fluorouracil (5-FU), resulting in cell death. Our protein-based technology is a modular system with genetically in-built specific targeting alongside delivery of protein payloads, allowing us to address a wide range of cellular functions such as selectively inducing cell death, regulating cellular pathways, and reprogramming gene expression. The purpose of this study is to show that self-assembling nanosyringes loaded with CD can be used to overcome some of the limitations associated with the CD/5-FC gene therapy system.

## Mechanism of action for nanosyringe-mediated delivery of biologics into cells



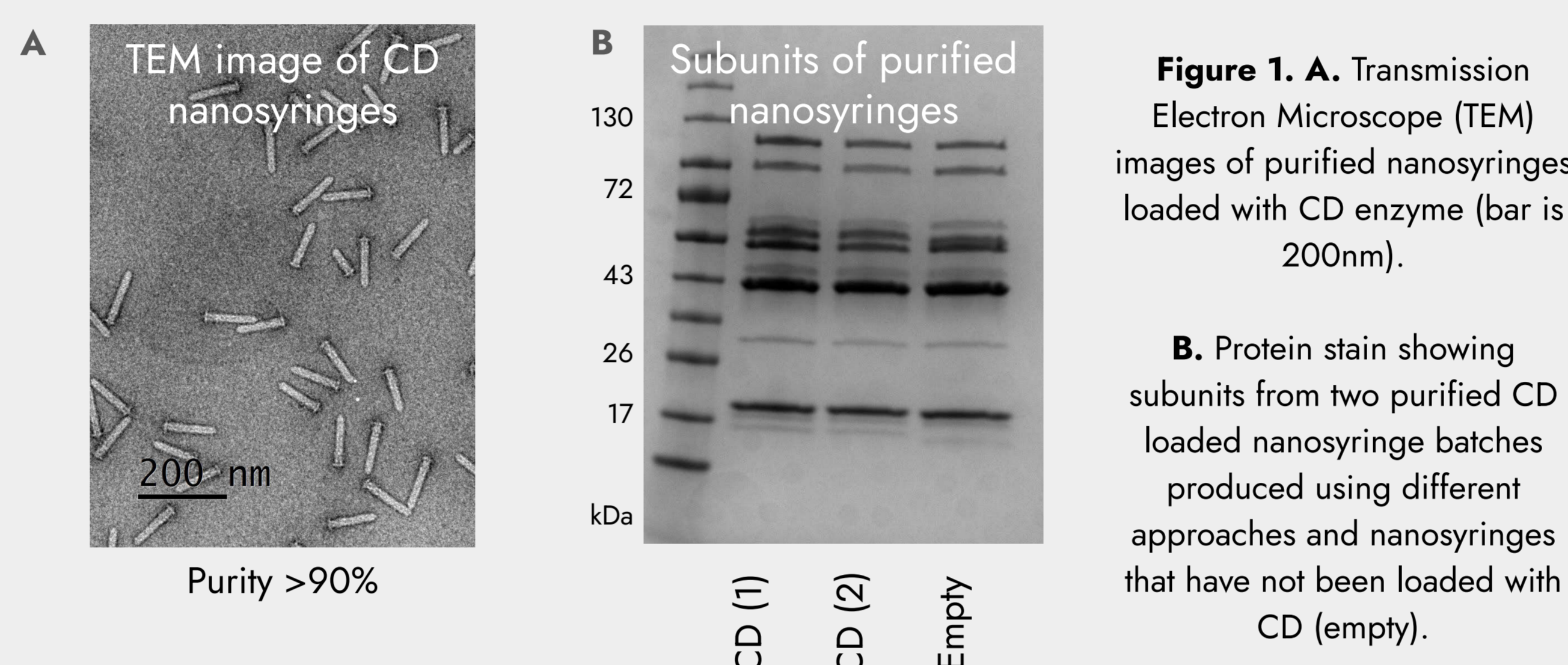
## Aims

- I. Produce nanosyringes loaded with *E. coli* cytosine deaminase (CD)
- II. Confirm binding of CD-loaded nanosyringes to target cells
- III. Show that nanosyringes can deliver functional CD enzyme into cells where it can convert inert 5-FC prodrug into cytotoxic 5-FU drug

## Methods

CD derived from *E. coli* was cloned into our nanosyringe plasmids. CD-loaded nanosyringes were then expressed in bacteria and purified using a number of approaches. Loading of the protein payload into the nanosyringes was confirmed by western blotting and quantified using ImageJ. Binding of CD-loaded nanosyringes to a murine macrophage cell line was assessed by flow cytometry. Cytotoxicity of 5-FC +/- CD-loaded nanosyringes and 5-FU in these cells was measured in triplicate using a LIVE/DEAD™ Viability/Cytotoxicity Kit. All cytotoxicity and payload quantification data was analysed and illustrated using GraphPad Prism.

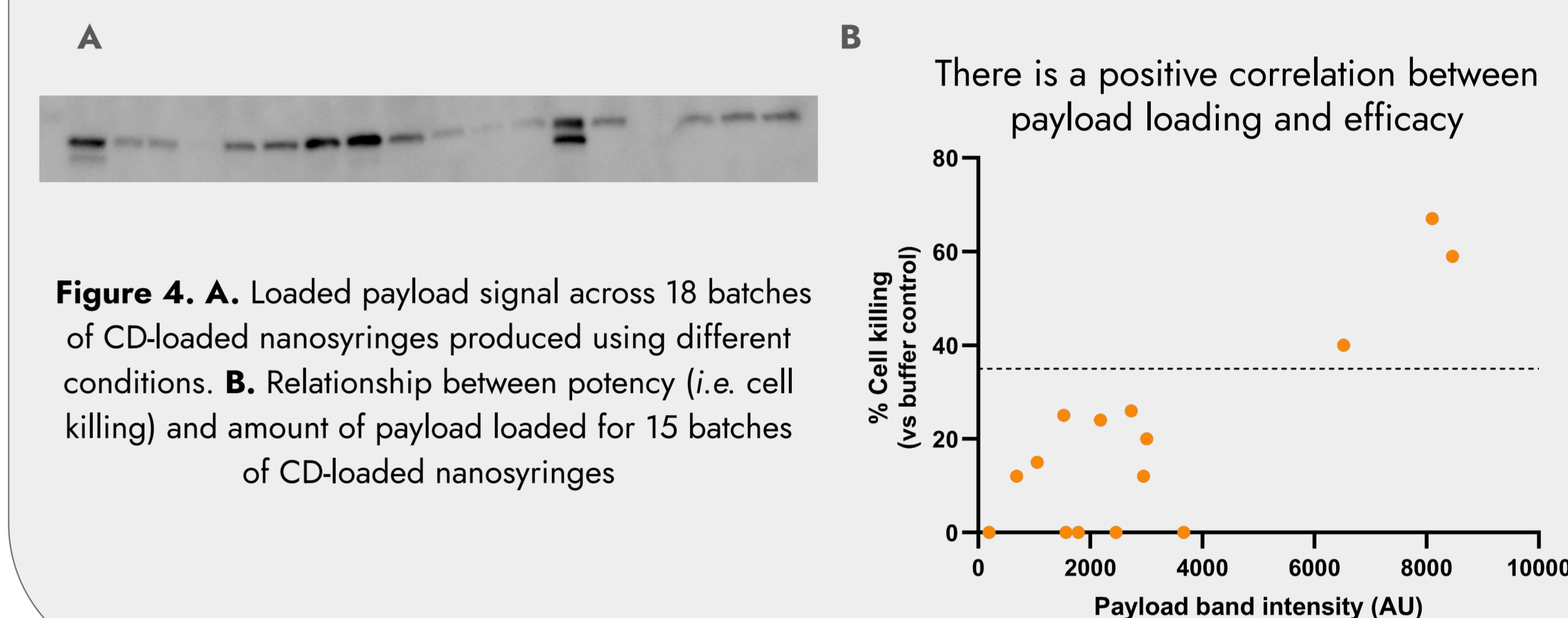
## Purification of CD-loaded nanosyringes



**Figure 1. A.** Transmission Electron Microscope (TEM) images of purified nanosyringes loaded with CD enzyme (bar is 200nm).

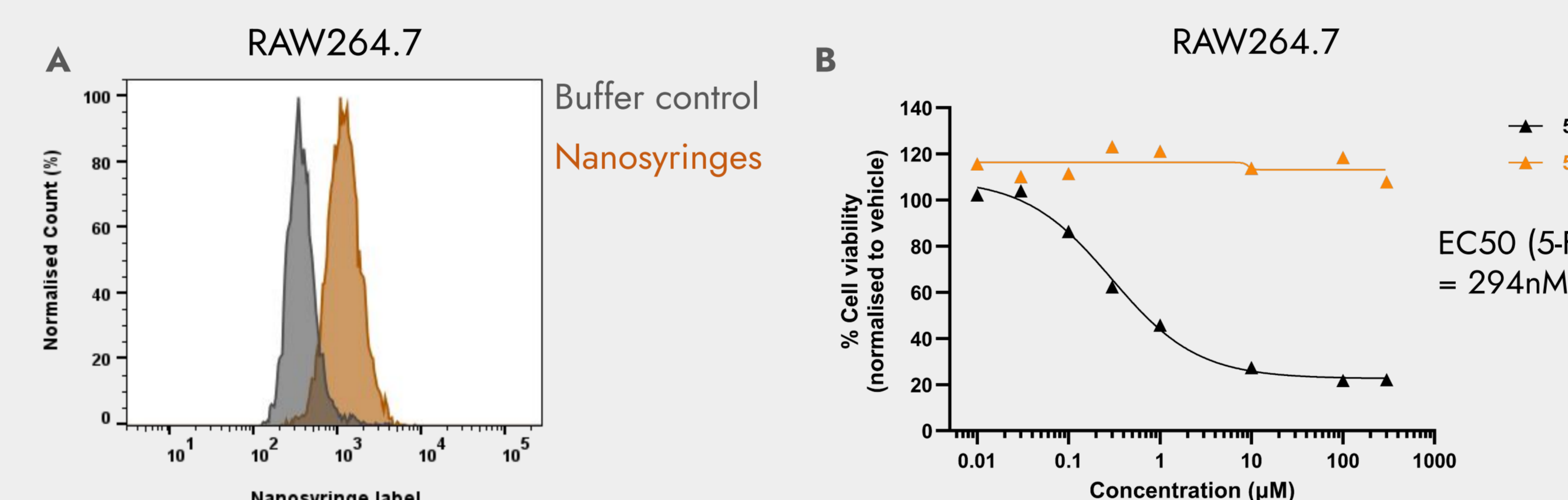
**B.** Protein stain showing subunits from two purified CD loaded nanosyringe batches produced using different approaches and nanosyringes that have not been loaded with CD (empty).

## Expression and purification conditions modulate the amount of payload loaded into nanosyringes



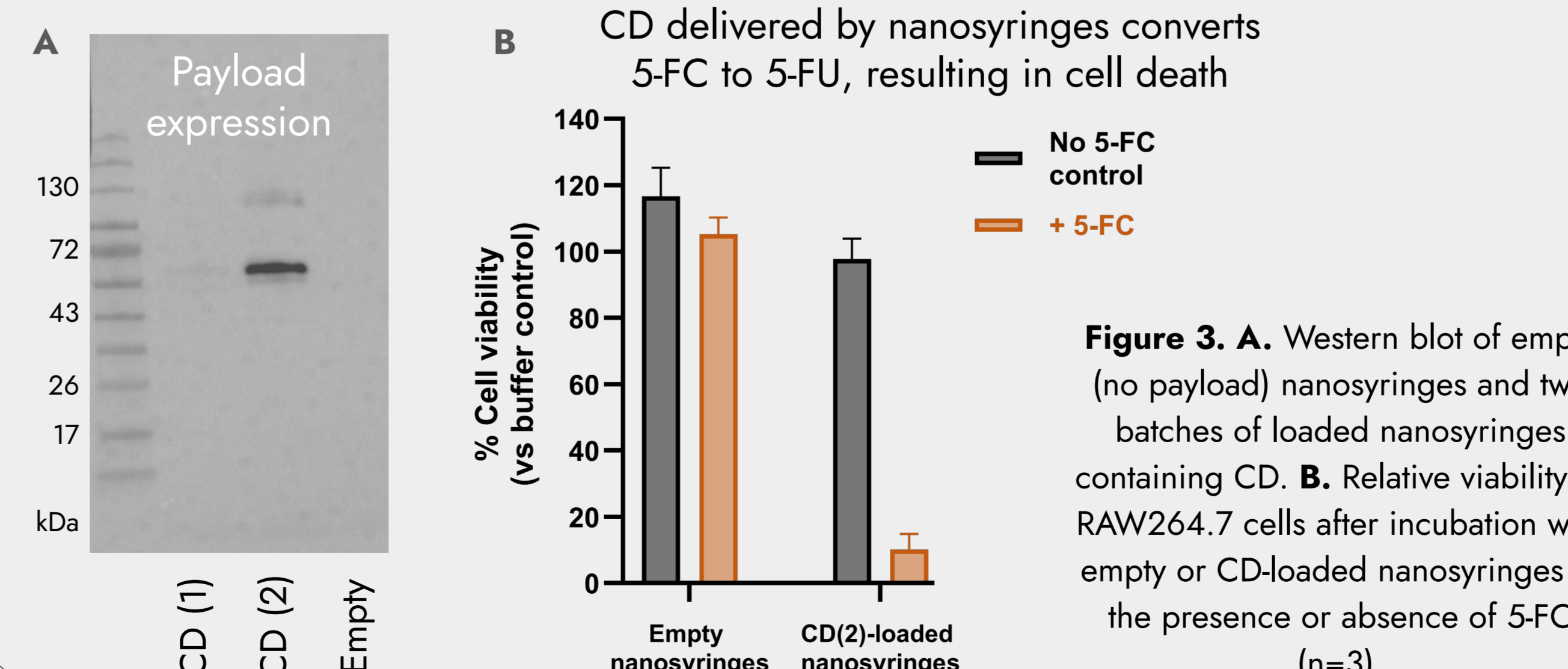
**Figure 4. A.** Loaded payload signal across 18 batches of CD-loaded nanosyringes produced using different conditions. **B.** Relationship between potency (i.e. cell killing) and amount of payload loaded for 15 batches of CD-loaded nanosyringes

## Nanosyringes bind to murine macrophages, which are susceptible to 5-FU, but not the 5-FC prodrug



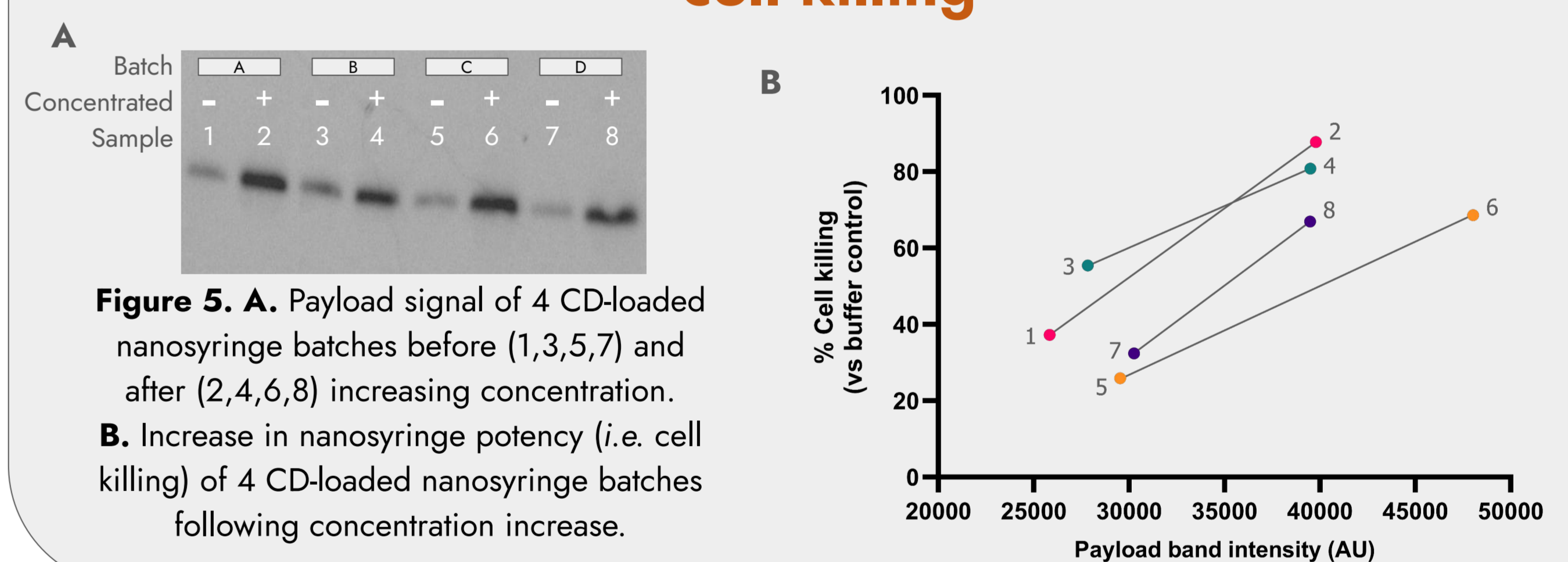
**Figure 2. A.** Flow cytometric analysis of mouse macrophages after incubation with labeled nanosyringes (orange) or buffer (grey). **B.** Susceptibility of mouse macrophages to 5-FU and 5-FC, T = 72h.

## Nanosyringes deliver loaded Cytosine Deaminase (CD) to murine macrophages, resulting in cell death



**Figure 3. A.** Western blot of empty (no payload) nanosyringes and two batches of loaded nanosyringes containing CD. **B.** Relative viability of RAW264.7 cells after incubation with empty or CD-loaded nanosyringes in the presence or absence of 5-FC (n=3).

## Concentrating the nanosyringes leads to increased cell killing



**Figure 5. A.** Payload signal of 4 CD-loaded nanosyringe batches before (1,3,5,7) and after (2,4,6,8) increasing concentration. **B.** Increase in nanosyringe potency (i.e. cell killing) of 4 CD-loaded nanosyringe batches following concentration increase.

## Conclusions

- I. We generated nanosyringes loaded with Cytosine Deaminase (CD) enzyme and showed that they bind to murine macrophages.
- II. We demonstrated that nanosyringes can deliver CD enzyme into cells where it assembles into its active, multimeric form and converts 5-FC prodrug into cytotoxic 5-FU, resulting in cell death. Nanosyringe expression and purification conditions directly modulated the amount of CD loaded into nanosyringes, which correlated with cell killing.
- III. Additional data with other payloads and nanosyringes targeted to cell membrane markers (not shown here) also highlights that expression and purification conditions can be optimised for loading specific payloads.

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